Title: The Inflammatory Effect of Iron Oxide and Silica Particles on Lung Epithelial Cells

Authors: Williams, L.J.[1] & Zosky, G.R.*[1]

Affiliation: [1] University of Tasmania, Australia

Address: 17 Liverpool St, Hobart TAS 7000

Corresponding author: Professor Graeme Zosky

Email: Graeme.Zosky@utas.edu.au

Phone number: +61 3 6226 6921

Acknowledgements: This work was funded by a Harry Windsor Grant from the Australian Respiratory Council

Word count for Abstract: 249

Word count for body of manuscript: 2597

Number of tables and figures: 5

Abstract

Purpose

Our understanding of the respiratory health consequences of geogenic (earth-derived) particulate matter (PM) is limited. Recent *in vivo* evidence suggests that the concentration of iron is associated with the magnitude of the respiratory response to geogenic PM. We investigated the inflammatory and cytotoxic potential of silica and iron oxide particles alone, and in combination, on lung epithelial cells.

Methods

Bronchial epithelial cells (BEAS-2B) were exposed to silica (quartz, cristobalite) and/or iron oxide (haematite, magnetite) particles. Cytotoxicity and cytokine production (IL-6, IL-8, IL-1β and TNF-α) were assessed by LDH assay and ELISA respectively. In subsequent experiments, the cytotoxic and inflammatory potential of the particles were assessed using alveolar epithelial cells (A549).

Results

After 24 hours of exposure, iron oxide did not cause significant cytotoxicity or production, nor did it augment the response of silica in the BEAS2-B cells. In contrast, while the silica response was not augmented in the A549 cells by the addition of iron oxide, iron oxide particles alone were sufficient to induce IL-8 production in these cells. There was no response detected for any of the outcomes at the 4 hour time point, nor was there any evidence of IL-1β or TNF-α production.

Conclusions

While previous studies have suggested that iron may augment silica induced inflammation, we saw no evidence of this in human epithelial cells. We found that alveolar epithelial cells produce pro-inflammatory cytokines in response to iron oxide particles, suggesting that previous *in vivo* observations are due to the alveolar response to these particles.

Introduction

Particulate matter (PM) inhalation is strongly associated with an increased risk of respiratory disease, cardiovascular disease and overall mortality [1-5]. The sources of PM vary considerably between locations. For example, urban populations are typically exposed to PM derived from combustion sources; in particular, diesel exhaust particles (DEP) which have been extensively studied due to their impact on the pathogenesis of respiratory disease [6,7]. In contrast, crustal, or geogenic (earth-derived) particles often affect populations in arid areas. Our understanding of the respiratory health impacts from these sources of PM is much more limited [8].

Inhalation of geogenic PM is associated with increased mortality [9-11] and hospital admissions [12]. In experimental models, inhalation of geogenic PM results in oxidative stress, release of pro-inflammatory mediators, reduced lung mechanics and exacerbation of viral infections [13-17]. *In vitro*, geogenic PM increases interleukin (IL)-6 and IL-8 production in bronchial epithelial cells [18] and tumour necrosis factor-α (TNF-α) and reactive oxygen species (ROS) in alveolar macrophages [19].

Oxides of silicon, aluminium and iron typically dominate geogenic PM. Silica (SiO_2) is well-known in the occupational setting for causing chronic lung disease [20] due its capacity to cause inflammation [21,22], cytotoxicity [23], DNA damage [24] and oxidative stress [25]. The effect of aluminium oxides on respiratory health is less well studied but the general consensus is that these particles are biologically inert when inhaled [26,27]. In contrast, data on the effect of iron oxides are contradictory. Epidemiologically, there is some evidence to suggest that exposure to iron oxide causes respiratory morbidity and *in vivo* studies have shown strong associations between the iron concentration in geogenic PM, inflammation, deficits in

lung mechanics and the capacity of the particles to exacerbate viral infection [17,16,15]. However, this is not always the case with some studies suggesting that insoluble iron oxides are biologically inert [28]. In contrast, some studies have suggested that the presence of particulate iron may synergistically enhance the silica induced respiratory response [29].

In light of the controversy regarding the effect of iron oxide laden particles on respiratory health *in vivo*, we investigated the inflammatory and cytotoxic potential of iron oxide (Fe_2O_3 and Fe_3O_4) particles, alone and in combination with silica, on lung epithelial cells to provide further insight into the potential health implications of inhalation of these particles.

Methods

Particle preparation

Commercially available standard preparations of dry magnetite (Fe_3O_4; Sigma-Aldrich 310069), haematite (Fe_2O_3; Sigma-Aldrich 310050), α-quartz (SiO_2; NIST 1878B) and cristobalite (SiO_2; NIST 1879A) were used. We assessed the effect of haematite (Fe^{2+}) and magnetite (Fe^{3+}) as the predominant forms of geogenic iron oxide. Particle samples were exposed to UV light for two hours to remove any endotoxin contamination.

Particle characteristics

See the online Supplement for details of the particle characterisation.

Cell culture

The transformed human bronchial epithelial cell line, BEAS-2B (ATCC CRL-9609), was cultured in 75 cm^2 flasks (Corning CLS3290), using serum-free bronchial epithelial growth medium (BEGM; Lonza CC-33170). The human lung alveolar epithelial cell line (A549; lung adenocarcinoma, ATCC CCL-185) was cultured in 75 cm^2 flasks (Corning CLS3290) with Ham's F-12K medium (Gibco 21127022), supplemented with 10% fetal bovine serum and 1% glutamine and antibiotics. Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2.

Cell exposure trials

Cells were seeded onto 12- and 96-well plates (Corning, CLS3512 & CLS3300) at a concentration of 1.9 x 10^5 cells/mL. To investigate the dose-dependent effects of iron oxide and silica individually, cells were exposed to 0 μg/mL, 0.38 μg/mL, 3.8 μg/mL, 19 μg/mL, 38 μg/mL or 57 μg/mL (0-15 μg/cm^2) of each particle type. Concentrations were chosen to be consistent with similar PM toxicology studies [30-34]. Cells were exposed for 4 or 24 hours.

Having established the dose dependent effects of the individual particle types, we then assessed the impact of silica and iron in combination on the response. Cells were exposed to either a 2:1 silica: iron ratio, which reflects the proportion of these elements in real-world particles [15], or a 20:1 ratio to replicate a situation where iron particles are present in trace amounts [35]. Having established the response in BEAS-2B cells, we then repeated a sub-set of experiments in the A549 alveolar epithelial cell line. We assessed a range of outcomes including cytotoxicity and cytokine production. All experiments were replicated in six independent trials conducted using fresh preparations of particle solutions and cell cultures to allow valid statistical comparisons between exposure groups.

Cytotoxicity

The lactate dehydrogenase (LDH) assay (Promega G1780) was used as a marker of cytotoxicity. LDH levels were measured after 24 hours of exposure according to the manufacturer's instructions. Briefly, 50 μL of LDH buffer was added to 50 μL of supernatant in a 96-well plate, incubated at room temperature and removed from light for 30 minutes. The absorbance was then read at 490 nm using the Spectra Max M2 plate-reader (Molecular Devices, USA).

Inflammatory cytokines

Inflammatory cytokines were assessed by enzyme-linked immunosorbent assay (ELISAs). We assessed levels of human interleukin-1β (IL-1β; R&D Systems DY201), interleukin-6 (IL-6; R&D Systems DY206), interleukin-8 (IL-8; R&D Systems DY208) and tumour necrosis factor-α (TNF-α; R&D Systems DY210) in the cell supernatant according to the manufacturer's instructions. The minimum detection limits for IL-1β, IL-6, IL-8 and TNF-α

were 7.81, 9.38, 31.3 and 15.6 pg/mL respectively. Plates were read using a Spectra Max M2 plate-reader (Molecular Devices, USA) at 450/570 nm absorbance.

Statistical analysis

Comparisons between groups were made using repeated measures one-way ANOVA. When significance was determined for the main factors by ANOVA, the Holm-Sidak post-hoc test was used to examine individual between group differences. Where necessary, the data were log transformed to satisfy the assumptions of normal distribution of the error terms and homoscedasticity of the variance. All data are presented as mean (SD) and values of $P < 0.05$ were considered statistically significant. All statistical analyses were conducted using SigmaPlot (v12.5).

Results

Assessment of particle structure

Cristobalite (Fig S1A) and quartz (Fig S1B) particle size ranged from 2 - 6 μm in diameter while Haematite (Fig 1C) and magnetite (Fig S1D) particle size ranged from 0.2-0.8 μm aerodynamic diameter. See online Supplement for further details.

Response to individual particles types (BEAS-2B)

Cytotoxicity

Exposure of BEAS-2B cells for 24 hours to cristobalite (Fig 1A, p= 0.017) or quartz (Fig 1B, p= 0.009) elicited an increase in LDH levels at 57 μg/mL compared to control. Haematite (Fig 1C, p = 0.392) and magnetite (Fig 1D, p = 0.708) had no effect on LDH levels following 24 hours of exposure. There was no change in LDH levels in response to any particle type 4 hours post exposure ($p > 0.05$) (*data not shown*).

Cytokines

Exposure for 24 hours to cristobalite (Fig 2A, p= 0.045) or quartz (Fig 2B, p= 0.009) elicited an increase in IL-6 levels at 57 μg/mL. Haematite (Fig 2C, p = 0.133) and magnetite (Fig 2D, $p > 0.250$) had no effect on IL-6 levels. There was no change in IL-6 levels in response to any particle type 4 hours post exposure ($p > 0.05$) (*data not shown*).

Exposure for 24 hours caused increased IL-8 for cristobalite at 38 μg/mL (Fig 3A, p= 0.031) and 57 μg/mL (Fig 3A, $p < 0.001$). Quartz elicited an increase in IL-8 levels at 57 μg/mL (Fig 3B, p= 0.011). Haematite (Fig 3C, p = 0.857) and magnetite (Fig 3D, p = 0.775) had no effect on IL-8 levels following 24 hours of exposure. There was no change in IL-8 levels in response to any particle type 4 hours post exposure ($p > 0.05$) (*data not shown*). Tumour necrosis factor-

α and interleukin-1β were measured, however all results were under the detection threshold (*data not shown*).

Combined effect of silica and iron oxide (BEAS-2B)

In initial experiments, described above, we determined the dose dependent cytotoxicity, cell metabolism and cytokine response to individual particle types. Subsequently, cells were exposed to combinations of particles to determine if the silica induced response was altered by the presence of iron oxide. For the combined exposure experiments, we chose to focus on the modifying effect of magnetite and hematite on the cristobalite induced response.

Cytotoxicity

When exposed for 24 hours, neither cristobalite-haematite (Fig 4A, $p = 0.096$) nor cristobalite-magnatite ($p = 0.253$) combinations elicited an increase in LDH levels in BEAS-2B cells above the cristobalite induced response.

Cytokines

38 μg/mL of cristobalite in combination with haematite (Fig 4B, 1.9 μg/mL $p= 0.005$ & 19 μg/mL $p= 0.04$) or magnetite (1.9 μg/mL $p= 0.011$ & 19 μg/mL $p= 0.012$) caused increased levels of IL-6 compared to controls when cells were exposed for 24 hours. However, neither the addition of haematite (Fig 4B, 1.9 μg/mL $p = 0.207$ & 19 μg/mL $p = 0.649$) nor magnetite (1.9 μg/mL $p = 0.933$ & 19 μg/mL $p = 0.890$) significantly increased the IL-6 response compared to 38 μg/mL of cristobalite alone.

38 μg/mL of cristobalite alone (Fig 4C, $p=0.021$) and in combination with either concentration of haematite (1.9 μg/mL $p < 0.001$ & 19 μg/mL $p = 0.001$) or of magnetite (1.9 μg/mL $p= 0.035$

& 19 μg/mL p= 0.037) caused increased levels of IL-8 when cells were exposed for 24 hours. However, neither the addition of haematite (Fig 4C, 1.9 μg/mL p = 0.207 & 19 μg/mL p = 0.246) nor magnetite (1.9 μg/mL p = 0.920 & 19 μg/mL p = 0.913) significantly increased the IL-8 response compared to 38 μg/mL of cristobalite alone. Tumour necrosis factor-α and interleukin-1β were measured, however all results were under the detection threshold (*data not shown*).

Combined effect of silica and iron oxide: the effect of cell type (A549)

Initial BEAS-2B experiments determined that both haematite and magnetite did not modify the silica-induced response. In order to test whether this observation is consistent in other cell types we also assessed the response in A549 cells, an alveolar type II epithelial cell line.

Cytotoxicity

There was no evidence of cytotoxicity in A549 cells in response to cristobalite and/or haematite (Fig 5A, p = 0.157) or magnetite (p = 0.106).

Cytokines

In contrast to the BEAS-2B cells, exposure to cristobalite (Fig 5B, $p < 0.001$) and haematite (p = 0.008), but not magnetite (p = 0.06), alone were sufficient to increase IL-8 levels. The combined effect of cristobalite and haematite was equivalent to the effect of the individual exposures (Fig 5B, p = 0.74). TNF-α, IL-1β and IL-6 were measured in the A549 cells, however all results were under the detection threshold.

Discussion

The present study aimed to investigate the effect of iron oxide, alone and in combination with silica, on the inflammatory response in respiratory epithelial cells to determine whether these cells are responsible for the observed association between iron content and the inflammatory response induced by geogenic particles observed *in vivo* [16,15]. Collectively, our data from BEAS-2B cells, a bronchial epithelial cell line, suggest that iron oxide has no effect on inflammatory cytokine production, nor do these particles exacerbate the silica-induced response. In contrast to the lack of response observed in the BEAS-2B cells, iron oxide particles induced IL-8 production in A549 cells; although they did not enhance the response induced by silica. Collectively, these data suggest that alveolar, but not bronchial, epithelial cells may be partly responsible for the association between the iron content and the inflammatory response to geogenic PM observed *in vivo* [15].

Using relatively low doses of particles compared to similar toxicological studies [36-38], we found that silica caused mild cytotoxicity and induced the production of IL-6 and IL-8 in BEAS-2B cells and IL-8 release in A549 cells. This is largely consistent with the wealth of literature on the known pro-inflammatory effect of silica [20] on BEAS-2B [22] and A549 cells [38]. There was no difference in the response between cristobalite and quartz, which is perhaps not surprising given the similarities in particle structure we observed. IL-1β and TNF-α release have long been associated with silica exposure in animal models [39,40]. Based on our data, secretion of these cytokines *in vivo* is most likely attributable to another cell type, such as macrophages [25,40,41].

In contrast, iron oxide, in the form of both haematite (Fe^{2+}) and magnetite (Fe^{3+}), was not cytotoxic at the doses used nor did it have any impact on the production of IL-6 and IL-8 by

BEAS-2B cells or the silica induced IL-6 and IL-8 response. However, while neither were cytotoxic in A549 cells, both iron oxides elicited IL-8 release. This is consistent with previous epidemiological studies showing a positive correlation between exposure to iron oxide laden PM and adverse health outcomes [42,43] but is inconsistent with previous studies suggesting that iron oxide PM may be relatively inert [28].

It is generally thought that any cellular damage induced by iron is driven by the Fenton redox reaction whereby Fe^{2+} is converted into Fe^{3+} and a hydroxyl radical is produced [44]. Theoretically, with prolonged exposure to Fe^{2+}, this results in excessive production of radical oxygen species. This requires the presence of free Fe^{2+} which is dependent on the solubility of the iron compound. However, free iron rarely exists in nature [45] and the common forms used in this study, haematite and magnetite are largely insoluble at physiological pH. This implies that without a catalyst, there is no dissociated Fe^{2+} and no potential for a Fenton-like reaction to occur. While it has not been determined whether the previously studied geogenic samples contained dissociated Fe^{2+}, Lay *et al.* (1999) suggest only small amounts of iron (0.036% dissociation) are necessary to produce significant amounts of radical oxygen species. It is unlikely that there was sufficient free iron in our system to induce this response. Given that it is unlikely that high enough concentrations of free iron were liberated in our cell culture system, the increase in cytokine production in the A549 cells suggests that this is a direct effect of the particles on the cells.

In accordance with our data, silica has previously been demonstrated to elicit IL-8 release in A549 cells [46]. There is some evidence to suggest magnetite can induce genotoxicity and cytokine release [47]. Interestingly, Konczol *et al.* (2011) saw no cytotoxicity or genotoxicity, which is consistent with our data. Of note is the fact that the combined effect of silica and iron

oxide on cytokine production was not greater than the effects of the individual particle types. It is likely that this is a threshold effect whereby the maximum production of IL-8 by these cells was reached.

IL-8 is a neutrophil chemoattractant and is key in recruiting neutrophils to a site of infection [48]. Recruitment of neutrophils results in endocytosis of invading pathogens and subsequent release of proteases and oxidant products [49]. Neutrophils naturally undergo autophagy, however, excessive or chronic IL-8 may lead to a disruption in the equilibrium of neutrophilic processes leading to excess and prolonged release of proteases and ROS and reduced anti-microbial function, which may result in damage to the lung tissue [50-52]. Our data suggests that exposure of alveolar cells to iron oxide containing particles may lead to tissue damage as a result of IL-8 production; an observation which is consistent with the long term deficits in lung function that are observed *in vivo* [15].

In summary, we found that iron oxide particles can induce an inflammatory response in alveolar epithelial cells, but appear to have no effect on bronchial cells. The iron oxide particles had no effect on the inflammatory response induced by silica, suggesting that the association between iron levels in geogenic particles and the inflammatory response *in vivo* is a direct effect of iron oxide. Collectively, these data highlight the importance of the iron oxide when considering the health implications of geogenic PM.

Conflict of Interest: None

References:

1. Lu F, Xu D, Cheng Y, Dong S, Guo C, Jiang X, Zheng X (2015) Systematic review and meta-analysis of the adverse health effects of ambient $PM_{2.5}$ and PM_{10} pollution in the Chinese population. Environ Res 136:196-204.

2. Pope III CA, Dockery DW, Spengler JD, Raizenne ME (1991) Respiratory Health and PM10 Pollution: A Daily Time Series Analysis. Am Rev Respir Dis 144:668-674.

3. Pope III CA, Dockery DW (1992) Acute Health Effects of PM10 Pollution on Symptomatic and Asymptomatic Children. Am Rev Respir Dis 145:1123-1128.

4. Medina S, Plasencia A, Ballester F, Mücke HG, Schwartz J (2004) Apheis: public health impact of PM in 19 European cities. J Epidemiol Community Health 58:831-836.

5. Brunekreef B, Holgate ST (2002) Air pollution and health. Lancet 360:1233-1242.

6. Steiner S, Bisig C, Petri-Fink A, Rothen-Rutishauser B (2016) Diesel exhaust: current knowledge of adverse effects and underlying cellular mechanisms. Arch Toxicol 90:1541-1553.

7. Ghio AJ, Sobus JR, Pleil JD, Madden MC (2012) Controlled human exposures to diesel exhaust. Swiss Med Wkly 142:w13597.

8. Williams LJ, Chen L, Zosky GR (2017) The respiratory health effects of geogenic (earth derived) PM10. Inhal Toxicol 29:342-355.

9. Perez L, Tobias A, Querol X, Künzli N, Pey J, Alastuey A, Viana M, Valero N, González-Cabré M, Sunyer J (2008) Coarse particles from Saharan dust and daily mortality. Epidemiology 19 (6):800-807.

10. Ostro BD, Hurley S, Lipsett MJ (1999) Air pollution and daily mortality in the Coachella Valley, California: a study of PM_{10} dominated by coarse particles. Environ Res 81 (3):231-238.

11. Mar TF, Norris GA, Koenig JQ, Larson TV (2000) Associations between air pollution and mortality in Phoenix, 1995-1997. Environ Health Perspect 108:347-353

12. Gyan K, Henry W, Lacaille S, Laloo A, Lamsee-Ebanks C, McKay S, Antoine RM, Monteil MA (2005) African dust clouds are associated with increased paediatric asthma accident and emergency admissions on the Caribbean island of Trinidad. Int J Biometeorol 49:371-376.

13. Wilfong ER, Lyles M, Rietcheck RL, Arfsten DP, Boeckman HJ, Johnson EW, Doyle TL, Chapman GD (2011) The acute and long-term effects of Middle East sand particles on the rat airway following a single intratracheal instillation. J Toxicol Environ Health 74:1351-1365.

14. Ghio AJ, Kummarapurugu ST, Tong H, Soukup JM, Dailey LA, Boykin E, Ian Gilmour M, Ingram P, Roggli VL, Goldstein HL, Reynolds RL (2014) Biological effects of desert dust in respiratory epithelial cells and a murine model. Inhal Toxicol 26:299-309.

15. Zosky GR, Iosifidis T, Perks K, Ditcham WGF, Devadason SG, Siah WS, Devine B, Maley F, Cook A (2014) The concentration of iron in real-world geogenic PM_{10} is associated with increased inflammation and deficits in lung function in mice. PLoS ONE 9:e90609.

16. Zosky GR, Boylen CE, Wong RS, Smirk MN, Gutierrez L, Woodward RC, Siah WS, Devine B, Maley F, Cook A (2014) Variability and consistency in lung inflammatory responses to particles with a geogenic origin. Respirology 19:58-66.

17. Clifford HD, Perks KL, Zosky GR (2015) Geogenic PM10 exposure exacerbates responses to influenza infection. Sci Total Environ 533:275-282.

18. Rodríguez-Cotto RI, Ortiz-Martínez MG, Rivera- Ramírez E, Méndez LB, Dávila JC, Jiménez-Vélez BD (2013) African dust storms reaching Puerto Rican Coast stimulate the

327 secretion of IL-6 and IL-8 and cause cytotoxicity to human bronchial epithelial cells (BEAS-
328 2B). Health 5:14-28.
329 19. Higashisaka K, Fujimura M, Taira M, Yoshida T, Tsunoda S-i, Baba T, Yamaguchi N,
330 Nabeshi H, Yoshikawa T, Nasu M, Yoshioka Y, Tsutsumi Y (2014) Asian dust particles induce
331 macrophage inflammatory responses via mitogen-activated protein kinase activation and
332 reactive oxygen species production. J Immunol Res 2014:856154.
333 20. Leung CC, Yu IT, Chen W (2012) Silicosis. Lancet 379:2008-2018.
334 21. Porter DW, Hubbs AF, Mercer R, Robinson VA, Ramsey D, McLaurin J, Khan A, Battelli
335 L, Brumbaugh K, Teass A, Castranova V (2004) Progression of lung inflammation and damage
336 in rats after cessation of silica inhalation. Toxicol Sci 79:370-380.
337 22. Perkins TN, Shukla A, Peeters PM, Steinbacher JL, Landry CC, Lathrop SA, Steele C,
338 Reynaert NL, Wouters EF, Mossman BT (2012) Differences in gene expression and cytokine
339 production by crystalline vs. amorphous silica in human lung epithelial cells. Part Fibre Toxicol
340 9:6.
341 23. Allison AC, Harington JS, Birbeck M (1966) An examination of the cytotoxic effects of
342 silica on macrophages. J Exp Med 124:141-154
343 24. Fanizza C, Ursini CL, Paba E, Ciervo A, Di Francesco A, Maiello R, De Simone P, Cavallo
344 D (2007) Cytotoxicity and DNA-damage in human lung epithelial cells exposed to respirable
345 alpha-quartz. Toxicol In Vitro 21:586-594.
346 25. Hamilton RF, Thakur SA, Holian A (2008) Silica binding and toxicity in alveolar
347 macrophages. Free Radic Biol Med 44:1246-1258.
348 26. Lindenschmidt RC, Driscoll KE, Perkins MA, Higgins JM, Maurer JK, Belfiore KA (1990)
349 The comparison of a fibrogenic and two nonfibrogenic dusts by bronchoalveolar lavage.
350 Toxicol Appl Pharmacol 102:268-281

27. Tornling G, Blaschke E, Eklund A (1993) Long term effects of alumina on components of bronchoalveolar lavage fluid from rats. Br J Ind Med 50:172-175

28. Lay JC, Zeman KL, Ghio AJ, Bennett WD (2001) Effects of inhaled iron oxide particles on alveolar epithelial permeability in normal subjects. Inhal Toxicol 13:1065-1078.

29. Castranova V, Vallyathan V, Ramsey DM, McLaurin JL, Pack D, Leonard S, Barger MW, Ma JY, Dalal NS, Teass A (1997) Augmentation of pulmonary reactions to quartz inhalation by trace amounts of iron-containing particles. Environ Health Perspect 105:1319-1324

30. Perkins T, Shukla A, Peeters P, Steinbacher J, Landry C, Lathrop S, Steele C, Reynaert N, Wouters E, Mossman B (2012) Differences in gene expression and cytokine production by crystalline vs. amorphous silica in human lung epithelial cells. Part Fibre Toxicol 9:6

31. Veranth J, Kaser E, Veranth M, Koch M, Yost G (2007) Cytokine responses of human lung cells (BEAS-2B) treated with micron-sized and nanoparticles of metal oxides compared to soil dusts. Part Fibre Toxicol 4:2

32. Longhin E, Holme J, Gutzkow K, Arlt V, Kucab J, Camatini M, Gualtieri M (2013) Cell cycle alterations induced by urban PM2.5 in bronchial epithelial cells: characterization of the process and possible mechanisms involved. Part Fibre Toxicol 10:63

33. Steenhof M, Gosens I, Strak M, Godri K, Hoek G, Cassee F, Mudway I, Kelly F, Harrison R, Lebret E, Brunekreef B, Janssen N, Pieters R (2011) In vitro toxicity of particulate matter (PM) collected at different sites in the Netherlands is associated with PM composition, size fraction and oxidative potential - the RAPTES project. Part Fibre Toxicol 8:26

34. Ramgolam K, Favez O, Cachier H, Gaudichet A, Marano F, Martinon L, Baeza-Squiban A (2009) Size-partitioning of an urban aerosol to identify particle determinants involved in the proinflammatory response induced in airway epithelial cells. Part Fibre Toxicol 6:10

35. Castranova V, Porter D, Millecchia L, Ma JY, Hubbs AF, Teass A (2002) Effect of inhaled crystalline silica in a rat model: time course of pulmonary reactions. Mol Cell Biochem 234-235:177-184

36. Peeters PM, Eurlings IMJ, Perkins TN, Wouters EF, Schins RPF, Borm PJA, Drommer W, Reynaert NL, Albrecht C (2014) Silica-induced NLRP3 inflammasome activation in vitro and in rat lungs. Part Fibre Toxicol 11:58.

37. ØVrevik J, Myran T, Refsnes M, LÅG M, Becher R, Hetland RB, Schwarze PE (2005) Mineral particles of varying composition induce differential chemokine release from epithelial lung cells: importance of physico-chemical characteristics. Ann Occup Hyg 49:219-231.

38. Hefland RB, Schwarzel PE, Johansen BV, Myran T, Uthus N, Refsnes M (2001) Silica-induced cytokine release from A549 cells: importance of surface area versus size. Hum Exp Toxicol 20:46-55.

39. Gossart S, Cambon C, Orfila C, Séguélas MH, Lepert JC, Rami J, Carré P, Pipy B (1996) Reactive oxygen intermediates as regulators of TNF-alpha production in rat lung inflammation induced by silica. J Immunol 156:1540-1548

40. Zhang Y, Lee TC, Guillemin B, Yu MC, Rom WN (1993) Enhanced IL-1 beta and tumor necrosis factor-alpha release and messenger RNA expression in macrophages from idiopathic pulmonary fibrosis or after asbestos exposure. J Immunol 150:4188-4196

41. Gozal E, Ortiz LA, Zou X, Burow ME, Lasky JA, Friedman M (2002) Silica-induced apoptosis in murine macrophage: involvement of tumor necrosis factor-alpha and nuclear factor-kappaB activation. Am J Respir Cell Mol Biol 27:91-98.

42. Western Australia Department of Health (2010) Impact of dust on Port Hedland. Western Australian Government.

43. South Australia Department of Health (2007) Whyalla Health Impact Study Report. South Australian Government.

399 44. Barbusiński K (2009) Fenton reaction - Controversy concerning the chemistry. Ecol Chem
400 Eng S 16:347-358
401 45. Cornell RM, Schwertmann U (2003) The iron oxides: structure, properties, reactions,
402 occurrences and uses. John Wiley & Sons.
403 46. Hetland RB, Schwarze PE, Johansen BV, Myran T, Uthus N, Refsnes M (2001) Silica-
404 induced cytokine release from A549 cells: importance of surface area versus size. Hum Exp
405 Toxicol 20:46-55.
406 47. Konczol M, Ebeling S, Goldenberg E, Treude F, Gminski R, Giere R, Grobety B, Rothen-
407 Rutishauser B, Merfort I, Mersch-Sundermann V (2011) Cytotoxicity and genotoxicity of size-
408 fractionated iron oxide (magnetite) in A549 human lung epithelial cells: role of ROS, JNK, and
409 NF-kappaB. Chem Res Toxicol 24:1460-1475.
410 48. Harada A, Sekido N, Akahoshi T, Wada T, Mukaida N, Matsushima K (1994) Essential
411 involvement of interleukin-8 (IL-8) in acute inflammation. J Leukoc Biol 56:559-564
412 49. Rosales C, Demaurex N, Lowell CA, Uribe-Querol E (2016) Neutrophils: Their Role in
413 Innate and Adaptive Immunity. J Immunol Res 2016:1469780.
414 50. Leliefeld PH, Wessels CM, Leenen LP, Koenderman L, Pillay J (2016) The role of
415 neutrophils in immune dysfunction during severe inflammation. Crit Care Med 20:73.
416 51. Newburger PE (2006) Disorders of neutrophil number and function. Hematology Am Soc
417 Hematol Educ Program:104-110.
418 52. Pham CTN (2008) Neutrophil serine proteases fine-tune the inflammatory response. Int J
419 Biochem Cell Biol 40:1317-1333.
420

Figure Legends:

Figure 1. LDH levels in the supernatant of BEAS-2B cells exposed to cristobalite (A), quartz (B), haematite (C) or magnetite (D) for 24 hours. Data are represented as a relative percentage increase in LDH optical density value compared to the control (100%). Data are presented as mean(SD) from 6 independent replicates with * indicating $p < 0.05$ versus control. Both cristobalite (A) and quartz (B) caused a significant increase in LDH, but only at a dose of 57 μg/mL ($p = 0.017$ & $p = 0.009$). Haematite (C; $p = 0.392$) and magnetite (D; $p = 0.708$) had no effect on LDH levels.

Figure 2. Interleukin-6 (IL-6) levels in the supernatant of BEAS-2B cells exposed to cristobalite (A), quartz (B), haematite (C) or magnetite (D) for 24 hours. Data are presented as mean(SD) from 6 independent replicates with * indicating $p < 0.05$ versus control. Both cristobalite (A) or quartz (B) caused a significant increase in IL-6, but only at a dose of 57 μg/mL ($p = 0.045$ & $p = 0.009$). Haematite (C; $p = 0.133$) or magnetite (D; $p = 0.250$) had no effect on IL-6 levels.

Figure 3. Interleukin-8 (IL-8) levels in the supernatant of BEAS-2B cells exposed to cristobalite (A), quartz (B), haematite (C) or magnetite (D) for 24 hours. Data are presented as mean(SD) from 6 independent replicates with * indicating $p < 0.05$ versus control. Cristobalite (A) caused a significant increase in IL-8 at doses of 38 μg/mL ($p = 0.031$) and 57 μg/mL ($p < 0.001$). Quartz (B) caused a significant increase in IL-8 but only at 57 μg/mL ($p = 0.011$). Both haematite (C; $p = 0.857$) and magnetite (D; $p = 0.775$) had no effect on IL-8 levels.

Figure 4. Supernatant of BEAS-2B cells exposed to cristobalite-haematite or cristobalite-magnetite combinations for 24 hours were assessed for relative LDH (A), IL-6 (B) and IL-8

(C). Data are presented as mean(SD) from 6 independent replicates with * indicating $p < 0.05$ versus control. Both cristobalite-haematite (Fig 4A; $p = 0.096$) and cristobalite-magnetite ($p = 0.253$) had no effect on LDH levels compared to cristobalite treatment. The addition of haematite or magnetite to 38 μg/mL of cristobalite caused an increase in IL-6. However, the addition of haematite (Fig 4B; 1.9 μg/mL $p = 0.207$ & 19 μg/mL $p = 0.649$) or magnetite (1.9 μg/mL $p = 0.933$ & 19 μg/mL $p = 0.890$) was not significantly greater than the response induced by 38 μg/mL of cristobalite alone. Likewise, the addition of haematite or magnetite to 38 μg/mL of cristobalite caused an increase in IL-8, however, the addition of haematite (Fig 4C; 1.9 μg/mL $p = 0.207$ & 19 μg/mL $p = 0.246$) or magnetite (1.9 μg/mL $p = 0.920$ & 19 μg/mL $p = 0.913$) was not significantly greater than the response induced by 38 μg/mL of cristobalite alone.

Figure 5. Supernatant of A549 cells exposed to cristobalite-haematite or cristobalite-magnetite combinations for 24 hours were assessed for relative LDH (A) and IL-8 (B). Data are represented as a relative percentage increase in LDH optical density value compared to the control (100%). Data are presented as mean(SD) from 6 independent replicates with * indicating $p < 0.05$ versus control. Both cristobalite-haematite (Fig 5A; $p = 0.157$)and cristobalite-magnetite ($p = 0.106$) had no effect on LDH levels. Cristobalite (Fig 5B; $p < 0.001$), haematite ($p = 0.008$), cristobalite-haematite ($p = 0.001$) and cristobalite-magnetite ($p < 0.001$) had significant effects on IL-8 levels.